

The intragenic approach as a new extension to traditional plant breeding

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The novel intragenic approach to genetic engineering improves existing varieties by eliminating undesirable features and activating dormant traits. It transforms plants with native expression cassettes to fine-tune the activity and/or tissue specificity of target genes. Any intragenic modification of traits could, at least in theory, also be accomplished by traditional breeding and transgenic modification. However, the new approach is unique in avoiding the transfer of unknown or foreign DNA. By consequently eliminating various potential risk factors, this method represents a relatively safe approach to crop improvement. Therefore, we argue that intragenic crops should be cleared through the regulatory process in a timely and cost-effective manner.

Introduction

Conventional plant breeding represents the principle approach to crop improvement. It employs methods such as introgression breeding, induced mutagenesis and somatic hybridization to modify randomly genomes and, as a result, create genetic variation (Figure 1a). Phenotypic assessments of segregating progenies can then identify the commercially important new traits that can be used to improve farm efficiency and enhance yield. However, today's crops are still a work-in-progress, and not all improvements can be delivered by breeding alone. One new method creates desired traits by isolating specific genetic elements from the crop, rearranging them *in vitro*, and inserting this 'intragenic' DNA back into the plant. This alternative approach to genetic engineering produces crops that, mimicking traditionally bred varieties, might be easier to commercialize than transgenic plants (see Glossary).

Issues associated with traditional plant breeding

Several key issues limit the potential of traditional methods in plant breeding to enhance quality and yield further. One drawback is based on the fact that genetic variation is induced at the DNA level but only screened for phenotypically. As a result, new cultivars not only contain traits that the breeder was looking for but also display

undesirable characteristics not considered during the selection process. Indeed, today's crops synthesize a multitude of natural pesticidal compounds and also often express dozens of allergen-encoding genes [1,2]. Although a few of the most important allergens were successfully removed through mutagenesis [3], the transfer of undesirable traits from existing to new varieties is generally viewed as inevitable.

A second issue is encountered as breeders intensify efforts to capture at least some of the genetic diversity that evolved within sexual compatibility groups (see Glossary). By performing wide crosses and extensively backcrossing interesting hybrids, they introgress new traits into cultivated varieties. These traits do not come alone but are embedded within much larger segments of wild chromosomes (so-called linkage drag). Assuming six backcrosses and random recombination, this uncharacterized DNA represents at least 1% of the entire genome and might contain hundreds of genes. Some of these new genes can be

Glossary

Famigenic plant: a transformed plant developed by transferring at least some DNA from one plant to a sexually incompatible plant that belongs to the same family.

Foreign genetic elements: elements such as genes, promoters or transfer DNA borders that did not evolve within the sexual compatibility group of the target plant.

Intragenic plant: a transformed plant that only contains genetic elements derived from within the sexual compatibility group.

P-DNA: a plant-derived transfer DNA that contains border-like elements and is used as alternative to the T-DNA.

Sexual compatibility group: the group of plant species that is able to exchange genetic material through interbreeding and represents the source of genetic material that is accessible to introgression breeding.

Species barriers: the physiological or biochemical barriers that prevent pairing or successful fertilization across different sexual compatibility groups.

Synthetic gene or xenogene: a gene that does not have a naturally evolved counterpart. In one example, the codons of a bacterial gene are replaced by codons that are more frequently used in a target crop to enhance translational efficiency. Another example relates to the PCR-based shuffling of related genes to produce variants that can then be selected for enhanced functional activity.

T-DNA: a DNA segment, delineated by *Agrobacterium*-derived left and right border regions, that can be transferred from a plasmid in *Agrobacterium* to plant cell nuclei.

Transgene: although initially used to indicate any gene that was introduced into a plant's genome through transformation, this term is currently often reserved for genes derived from a different family.

Transgenic plant: a transformed plant containing DNA from a different plant family.

Xenogenic plant: a transformed plant carrying synthetic DNA.

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	Source Genetic distance				Development time		Issues					Trait potential	Regulation (proposed)
	Xenogenic	Transgenic	Famigenic	Intragenic	~4-6 years	~8-20 years	% genome	Transfer of unknown DNA	Genetic complexity	Transfer of foreign DNA	Public concerns		
(a) Traditional breeding													
				X				+	+	-	-	Transfer of existing traits (native genes) from one to another variety.	Basic
				M0			>1%	+	+	-	-	Modification of existing traits through constitutively altered gene expression (generally knock outs).	Basic
				F1 hybrid			>1%	+	+	-	-	Introduction of new traits that are similar to existing traits and often associated with disease or stress tolerance.	Basic
(b) Genetic engineering													
				Somatic hybrid			>1%	+	+	+	*	Introduction of new traits that are similar to- but possibly stronger than existing traits, and often associated with disease or stress tolerance.	Full
				Binary vector			<0.1%	-	-	+	+	Introduction of new traits that may outperform native traits by transforming plants with genes from viral, bacterial, fungal or unrelated plant sources.	Full
				P-DNA vector			<0.1%	-	-	-	?	Creation of desired traits by fine-tuning the expression of native genes, often in a tissue-specific manner.	Basic
							<0.1%	-	-	+	?	Transfer of traits from related but sexually-incompatible species by transforming plants with genes that are linked to their own promoters.	Dep.

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Figure 1. Summary of various methods for crop improvement. The genetic distance between DNA source and target crop is indicated in the left four columns with red, referring to 'foreign', and green, indicative for 'sexually compatible'. The species barrier is shown at dotted vertical line. Xenogenic = synthetic DNA; transgenic = DNA from unrelated species, such as viruses, bacteria, fungi and plants that belong to different families; famigenic = DNA from plants that belong to the same family; and intragenic = DNA from within the same sexual compatibility group. The time to develop a new variety is indicated in yellow columns with mutation breeding, introgression breeding and somatic hybridization usually requiring 8 to 20 years. Development of transformed potato varieties requires ~four months for transformation and three years for propagation, line selection, and bulk up. Most other genetically modified crops require additional short backcross breeding programs that can extend timelines by two to three years. The grey column shows the estimated size of the introduced DNA as percentage of the entire genome. Introgression will often result in transfer of at least 1% of wild DNA although this percentage can in exceptional cases be lower. The amount of DNA that is introduced through transformation is generally smaller than 0.1% of the genome (a 10-kb transfer DNA represents 0.1% of the relatively small potato genome). F1 hybrids derived from interspecies somatic hybridization might need to undergo γ -radiation to overcome suppressed recombination. The asterisk indicates that there are some public concerns about interspecies somatic hybridization in Europe. Proposed regulatory requirements are shown in bold letters with 'Basic' implying multi-year field tests on agronomic performance and an assessment of the nutritional profile, and 'Full' indicating more extensive studies, which include biosafety assessments of foreign proteins as well as environmental studies. Regulatory requirements for cisgenic

involved in the production of new toxins or allergens, or otherwise negatively affect the quality of a crop. For instance, transfer of 'high starch' and 'crisp chip' traits from *Solanum chacoense* to cultivated potato (*Solanum tuberosum*) produced the commercial variety Lenape, which, after its release, was found to produce almost twice the maximum allowed concentration of toxic glycoalkaloids [4,5]. The use of molecular marker strategies accelerates the introgression process and aims to limit the amount of wild DNA [6] but does not address the potential safety issues associated with the transfer of uncharacterized genes. Transfer of genes of interest from sexually incompatible species by interspecies somatic hybridization through protoplast fusion results in even more complex mixtures of native and uncharacterized genes. In Figure 1a, we have outlined the characteristics of traditional breeding and the associated issues, highlighting that there are no public concerns associated with these approaches in the United States and most other countries.

The third limitation results from the inability of breeding to readily fine-tune expression of target genes in a tissue-specific manner. Many genes play an important role in certain tissues but can induce deleterious effects in others. For instance, genes involved in the degradation of starch are essential for both energy production and sugar signal transduction that controls plant growth and development. In potato tubers, however, expression of these genes produces undesirable sugars that react with amino acids during heat processing. The resulting Maillard products darken French fries and potato chips, and include toxic compounds, such as the carcinogen acrylamide [7]. Efforts to inactivate the starch degradation genes through plant breeding generally result in knockouts that display substantially reduced yields. A different example relates to attempts to increase the levels of essential amino acids or health-promoting compounds. Instead of overproducing these compounds in the edible parts of a crop only, conventional breeding often produces plants that display new constitutive phenotypes linked to reduced yield [8–10].

We conclude that traditional methods in plant breeding will continue to develop new and improved varieties. However, these methods are, by themselves, not sufficient to unleash fully the plant's own potential in terms of yield and quality.

Benefits of transgenic and xenogenic plants

Genetic engineering is different from the traditional methods in that any modification can be designed and tailored to achieve the desired effect. This method often fuses promoters and genes to produce expression cassettes that are introduced into plants using bacterial transfer DNAs (T-DNAs; see Glossary) (Figure 1b). It excludes the transfer of known allergen- or toxin-encoding genes and analyzes the sequence of insertion sites. The ability to identify rapidly and eliminate plants containing inadvertent fusions or disruptions of genes is not available to

traditional plant breeding, where genes can be inactivated through unpredictable transposition of resident mobile elements.

The second advantage of transgenic applications is that it generally takes less than a year to transform an existing variety with one or several traits. Subsequent line selection, bulk-up, and, in some cases, limited crossing/backcrossing programs only require an additional three to five years. Furthermore, several new traits can be introduced as a unit that segregates as single dominant locus. These linked traits are more easily transferred to other varieties than the often complex unlinked loci identified by traditional methods.

The option to transform plants with foreign genes overcomes species barriers (see Glossary), making it possible to exploit powerful 'super-traits' that are not attainable through traditional methods. One example of a crop carrying such new characteristics is Monsanto's multi-stacked maize, which was produced via conventional crossing of three inbred transgenic maize lines: MON863, MON810 and NK603. The elements incorporated into this multistack include five loci, four of which carry a synthetic gene (see Glossary) linked to combinations of strong regulatory elements from viruses, bacteria and unrelated plants. Expression of the first two synthetic genes produces a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) that resembles the EPSPS from *Escherichia coli* and is, unlike most plant versions, not inactivated by herbicides containing glyphosate. The third synthetic gene encodes the insecticidal cry3Bb1 protein with activity against specific Coleoptera, whereas the fourth gene product, cry1Ab, provides tolerance against certain Lepidopteran insects. The fifth gene is a bacterial kanamycin resistance gene encoding neomycin phosphotransferase (nptII). The pentuple stack maize currently occupies 5.8 million acres in the United States, and supports a substantial reduction in pesticide usage.

Issues arising from the commercial production of transgenic and xenogenic crops

Although expression of foreign genes can lower input costs while increasing yields, it is critical to evaluate carefully foreign genes because their expression in crops can trigger unexpected events. The new proteins can, for instance, represent entirely new classes of allergens or produce, directly or indirectly, new toxins that are not immediately recognized as harmful [11]. Measures to evaluate the risk of novel proteins are an integral part of the deregulation process, and include analyses of the crop's nutritional profile and potential safety risks to the environment ([12]; http://www.aphis.usda.gov/brs/brs_usersguide.html). The resulting complexity of the regulatory process has worked well for commodity crops, but often represents a cost-prohibitive barrier to commercialization for vegetables and fruits that occupy relatively small market niches (<http://pewagbiotech.org/events/0602>). Direct compliance costs, which only

applications are dependent on the trait ('Dep.'). In these cases, the transfer of traits that resemble native traits, such as those associated with disease resistance, should be considered for the basic regulatory assessment described above. However, traits that are new to the sexual compatibility group would require more extensive analyses. (a) Methods in traditional breeding. 'M0' stands for an original plant derived from induced mutagenesis. Random mutations are shown as dark green triangles, and can represent hundreds of point mutations/chromosome induced by ethylmethane sulphonate (EMS) or deletions of up to 100 kilobase pair triggered by diepoxybutane (DEB) or low linear energy transfer radiation (LET). (b) Methods in genetic engineering. 'Tn' stands for plant transformation.

represent part of the regulatory burden, were recently estimated at 6 to 15 million US dollars [13].

Public perception represents an additional issue that is associated with the transgenic or xenogenic engineering of food crops. In contrast to traditional methods that dramatically affect genome integrity, such as introgression breeding, mutation breeding and somatic hybridization, genetic engineering continues to spark consumer concerns. It has been hypothesized that this apprehension is related to the stable introduction of foreign DNA into food crops rather than to the modifications of plant genomes *per se* [14–16]. Even in the United States, public support for genetic engineering is still at the same low levels (26–27%) as in 2001 (<http://pewagbiotech.org>). This lukewarm support provides the backdrop for non-governmental organizations (NGOs) such as Greenpeace to discourage successfully the production and sale of genetically engineered specialty crops. In 2003, Nielsen proposed to bridge the gap between agricultural biotechnology companies at one side, and consumers and NGOs at the other side by diversifying genetically modified crops based on the genetic distance between DNA source and target crop [17]. He defined organisms transformed with native DNA as intragenic, while using the term famigenic for plants containing DNA from the same family. Nielsen considered plants containing DNA from unrelated sources as transgenic and labeled most currently available GM crops as xenogenic (Figure 1b) because they contain synthetic genes that lack naturally evolved counterparts.

Two preliminary surveys in the United States seem to confirm that the debate about genetic engineering is linked to the extent to which modified organisms differ from traditionally bred varieties. Whereas ~77–81% of respondents would accept a vegetable that contains an extra gene from that same vegetable, only 17–25% would be willing to consume a food that is transformed with a bacterial gene [18,19]. An independent, unpublished study performed by Scott Smith (Qualtrics, Inc) that was based on an email survey of 779 consumers confirmed these findings, with 70% indicating that intragenic modifications were an acceptable means of producing improved vegetables, versus 26% support for transgenic modifications. Genetic modification garners even more public support if the resulting products provide clear and transparent benefits to consumers [20].

The transgenic and xenogenic approaches have become a reality for the improvement of commodity crops. The use of these new plants and their super-traits makes it possible to increase farm efficiency, lower pesticide usage and increase yield [21]. However, regulatory costs, consumer concerns and pressure from NGOs have slowed application of these methods in specialty crops such as vegetables, fruits, nursery plants and trees.

The intragenic approach

One new method that combines the benefits of traditional breeding and genetic engineering, but circumvents many of their issues, is represented by the intragenic approach [22,23]. It isolates specific genetic elements from a plant, recombines them *in vitro*, and inserts the resulting expression cassettes into a plant that belongs to the same sexual

compatibility group using plant-derived transfer DNAs (P-DNAs; see Glossary) and marker-free transformation [23,24] (Figure 1b). The intragenic method does not offer xenogenic super-traits, but does not incorporate unknown or foreign DNA into a plant's genome. Details and benefits of this method are described in the following sections.

The intragenic method

There are four requirements for the transformation of plants with only native DNA [23]. First, the native target gene needs to be linked to suitable regulatory elements. Although tissue-specific promoters required for most intragenic modifications are available for well-characterized crop systems such as tomato, potato, canola and maize, it can be necessary to isolate new genetic elements from plants for which there are no extensive molecular or genomic data, as is the case with, for instance, melon and onion. Various polymerase chain reaction-based methods are available to obtain such native elements efficiently [25]. Second, the resulting expression cassettes need to be inserted into species-specific P-DNAs to circumvent the need of using bacterial T-DNAs. These vehicles for gene transfer were developed for crops such as tomato, potato, canola, alfalfa, apple, barley and rice, and can be isolated from other crops using recently published guidelines [24]. Third, marker-free transformation systems are necessary to introduce the expression cassette into the plant genome without the burden of foreign or unwanted DNA. One method that is applicable to dicotyledonous plant species co-transfers a P-DNA with a second 'Life-Support' transfer DNA that contains two selectable marker genes [22]. After selection for transient expression of the first marker followed by selection against stable integration of the second marker, plants are produced that frequently only contain the P-DNA. Alternative marker-free transformation systems can be used as well [26–28].

Examples of intragenic modification

The intragenic method was applied to produce a quality-enhanced potato [29]. This potato contains a P-DNA harboring a construct for tuber-specific silencing of both the polyphenol oxidase (*Ppo*) 'black spot bruise' gene and the two starch degradation-associated *R1* and phosphorylase-L (*PhL*) genes. The modification improved tuber quality in several different ways: elimination of black spot bruise and reduced sugar ends boosted the visual appeal of processed potato products, whereas lower cold-sweetening was associated with enhanced fry flavor, reduced amounts of processing-induced acrylamide, and increased starch levels.

Intragenic methods are currently being used to develop bruise-tolerant apples by transforming them with apple-derived P-DNAs carrying *Ppo*-gene silencing cassettes (www.okanaganbiotechnology.com). Another ongoing project develops drought-tolerant ryegrass (*Lolium perenne*) that overexpresses a native *Avp1*-like salt-tolerance gene (www.isb.vt.edu/articles/aug0601.htm). Additional examples of intragenic modification are often still theoretical, with efficacy demonstrated by transgenic experiments (Table 1). For instance, overexpression of biosynthetic genes can boost vitamin, flavonoid and carotenoid levels

Table 1. Examples of currently available native traits

Class	Trait	Approach	Refs
Health-promoting traits	High flavonols	<i>Chi</i> overexpression ^a	[39]
	High anthocyanins	<i>Ant1</i> overexpression	[40]
	High carotenoids	<i>Lcy-e</i> silencing	[41]
	High chlorogenic acid	<i>Cai</i> overexpression	[42]
	High vitamin C	<i>GalUR</i> overexpression ^a	[43]
	High vitamin E	<i>Vte3</i> + <i>Vte4</i> overexpression ^a	[44]
	Increased amylose/amylopectin ratio	<i>Sbel</i> + <i>Sbell</i> silencing	[45]
	Increased folate	<i>Acads</i> overexpression	[46]
	Enhanced oil stability	<i>Fad2</i> silencing	[47]
	Reduced allergen content	<i>Gly m Bd 30 K</i> silencing	[48]
	Reduced toxin content	<i>R1</i> + <i>PhL</i> silencing	[29]
Consumer traits	Enhanced aroma	<i>Cgs</i> overexpression ^a	[49]
	Enhanced flavor	<i>R1</i> + <i>PhL</i> silencing	[29]
	Bruise tolerance	<i>Ppo</i> silencing	[29]
	Extended shelf life	<i>Pg</i> silencing	[50]
Input traits	Late blight resistance	Transfer of <i>RB</i> ^b	[51]
	Sulfonylurea tolerance	Modified <i>Als</i> overexpression	[52]
	Salt tolerance	<i>Nhx1</i> overexpression	[53]
	Freezing and drought tolerance	<i>Cbf</i> overexpression ^a	[54]
Feed value	Reduced lignin	<i>C4h</i> silencing	[55]

^aTarget crops contain functional homologs of the genes from foreign plants that were used to demonstrate the trait concepts.

^bThe *RB* gene is derived from a wild potato species that is not sexually compatible with cultivated potato.

in a tissue-specific manner. Furthermore, intragenic silencing approaches can downregulate the expression of undesirable genes. Most allergen proteins in plants are present as isoforms encoded by genes that are members of multigene families. Therefore, silencing constructs carrying fragments of genes, each of which represents a different family, could be used to simultaneously downregulate the expression of multiple allergen-encoding genes [30].

Using only native DNA in crop modification can carry its own complexities that need to be optimized on a case-by-case basis. For example, the introduction of an extra copy of a native promoter or gene intended to increase expression levels might inadvertently trigger gene silencing. This phenomenon can be circumvented by employing chromosome boundary domains [31]. Another issue is that native genes are in some cases more difficult to overexpress than foreign genes. To increase the abundance of endogenous proteins regulated by negative feedback mechanisms, genes encoding enzymes such as the threonine synthase and aspartate kinase must be modified to reduce the protein's feedback sensitivity [32].

Marker-free methods are also being used to mobilize genes between related species to create famigenic crop improvements. Efforts at the Sainsbury's Laboratories (UK), Wageningen University (Netherlands) and USDA/ARS (USA) are independently seeking famigenic transfer of the late blight disease resistance genes from *Solanum bulbocastanum* to domesticated potato (J. Jones and W. Belknap, personal communication). This particular application does not require a modification of gene expression levels, and is referred to as cisgenesis [33; Figure 1b].

Intragenic crops are at least as safe as those developed through traditional methods

Intragenic modifications improve the agronomic performance or nutritional characteristics of crops but do not introduce traits that are new to the sexual compatibility group. As discussed above, intragenic plants (see Glossary) lack new unknown DNA that might comprise

genes associated with the production of toxins, allergens or antinutritional compounds. The plants also lack selectable marker genes, powerful insecticidal genes or any other foreign genes that are new to agriculture or the food stream. Furthermore, the modified expression levels of one or several native genes are not expected to trigger a phenotypic, biochemical or physiological variation that is not already present in the sexual compatibility group. One argument for this assertion is that any modification accomplished through all-native DNA transformation could, at least theoretically, be created by conventional breeding. Whereas single translocation events in traditional breeding would produce cisgenic plants [33], intragenic modifications mimic the effect of multiple translocations. Furthermore, any intragenic modification of gene expression levels is likely to fall within the extensive allele-specific differences that evolved naturally. For instance, 6–15% of *Arabidopsis* genes are differentially expressed by any tested pair of ecotypes [34]. At one end of the spectrum are the knockout (loss-of-function) mutations, which can be isolated for many non-essential genes in natural populations and are obtained at higher frequency using either natural or chemical mutagens. Individuals with enhanced gene expression, at the other end of the spectrum, can be recovered during plant selection, such as those adapted to specific environmental stresses [35]. Both classes yield rare phenotypes pursued by breeders that can often be developed using intragenics. In a targeted analysis of important compounds and metabolites in transgenic potato tubers with modified primary carbohydrate metabolism, polyamine biosynthesis, and glycoprotein processing demonstrated that there were no consistent differences with respect to appropriate controls [36]. Broader scale metabolomics analyses reached a similar conclusion, as did proteomic analysis [37,38].

Creation of unexpected compounds is an oft-cited fear of plant modification, even if the gene is endogenous. However, any increase in the level of one or several vitamins, minerals, or other dietary components that is intragenically-induced

remains within the limits set by the species itself and is, as discussed, not associated with the potentially undesirable consequences of transferring unknown DNA. We conclude that the potential risk of undesirable effects triggered through altered expression levels of a target gene is lower than that for plants developed through broadly accepted methods such as introgression- and mutation breeding. New varieties developed through any of these three methods represent low risk crops that should undergo a similar timely and cost-effective regulatory process. For example, while a case by case approach remains the pragmatic option, approval for release should not require extensive studies on potential environmental effects but rather focus on nutritional equivalence and absence of new toxins or allergens. By contrast, the expression of foreign genes in transgenic or xenogenic plants (see Glossary) would require more in depth studies to ascertain that the new proteins neither compromise food quality nor affect fitness in ways new to the species. In addition to addressing these potential safety risks, it is important to also consider the distance between gene source and target crop as part of the regulatory process. Disclosure of the sources of the genetic material introduced may prove necessary to define further research directions, maintain product identity, and increase consumer familiarity through categorization, and thus improve the response to engineered organisms and their products [17].

American regulatory agencies are currently considering revamping the approval process by assigning new modified products and crops into risk categories (<http://www.aphis.usda.gov/brs/eis/index.html>). If assigned as low risk, intragenic technologies could be readily applied for numerous improvements of specialty crops (<http://pewagbiotech.org/events/0118/WorkshopReport.pdf>). However, categorized risk assessments are not yet considered in the European Union. A desirable international harmonization of the regulatory process would require further debate.

Conclusion

The numerous methods in crop improvement all have their benefits and limitations, and will likely be employed whenever most suitable. Traditional methods will provide the baseline material that contains important combinations of traits. Genetic engineering can then be used to eliminate undesirable features while enhancing positive traits. Transgenic and xenogenic methods will mainly be applied to introduce powerful new traits into commodity crops, whereas intragenic and famigenic methods may provide more cost-effective and acceptable means for the improvement of specialty crops.

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